



DNA methylation and cancer therapy

Moshe Szyf*

*Department of Pharmacology and Therapeutics, McGill University, 3655 Sir William Promenade,
Montreal, Que., Canada H3G 1Y6*

Received 1 October 2003; received in revised form 10 October 2003; accepted 10 October 2003

Abstract

Vertebrate DNA is modified by methyl moieties at the 5'-position of cytosine rings residing in the di-nucleotide sequence CpG. Approximately 80% of CpG dinucleotide sequences are methylated. The pattern of distribution of methylated CGs is cell-type specific and correlates with gene expression programming and chromatin structure. Three kinds of seemingly contradictory aberrations in DNA methylation are observed in cancer, global hypomethylation, and regional hypermethylation and deregulated level of expression of DNA methyltransferases. It was previously proposed that the DNA methylation machinery is a candidate target for anticancer therapy. Inhibition of hypermethylation was the first therapeutic target. However, recent data suggests that inhibition of DNA methylation might have untoward effects such as induction of genes involved in metastasis. This review discusses the relative role of the three levels of alteration in the DNA methylation in cancer, proposes a unified hypothesis on the relative roles of increased DNA methyltransferase as well as the coexistence of hypo- and hyper-methylation in cancer and its possible implications on anticancer therapy.

© 2003 Elsevier Ltd. All rights reserved.

Keywords: DNA methylation; DNA methyltransferase; Chromatin structure; Histone acetylation; Histone methylation; Histone deacetylases; DNA demethylase; Global hypomethylation; Methylated DNA binding proteins; Gene silencing; Epigenomic imprint

1. Patterns of DNA methylation

Cytosines found in the di-nucleotide sequence CpG are methylated in vertebrate DNA (Razin and Riggs, 1980). A hallmark of DNA methylation in vertebrate DNA methylation is the fact that not all CpG dinucleotides are methylated and that non-methylated CpG dinucleotides are distributed in a cell-type specific manner creating cell-type specific patterns of methylation (Razin and Szyf, 1984). Early observations suggested that the distribution of methylated DNA correlated with the distribution of active and inactive chromatin in the nucleus (Razin and Cedar, 1977). Methylated DNA is associated with inactive chromatin while active chromatin associates with hypomethylated DNA (Razin and Cedar, 1977). Later studies revealed that regulatory regions of inactive genes are frequently methylated. This led to the hypothesis that DNA methylation is involved in silencing gene expression (Razin and Riggs, 1980; Razin and Shemer, 1999; Razin, 1998). Thus, the pattern of methylation reflects the gene expression profile of a cell.

The tight relation of DNA methylation, chromatin structure and gene expression begs the question of whether DNA methylation plays a causal role in gene expression or whether it is a consequence of either the state of either chromatin structure or gene expression. In any case, DNA methylation patterns are a true reflection of the state of activity of a gene and therefore serve as important diagnostic markers. Profiling DNA methylation patterns of different tumors using high throughput methylated DNA micro-arrays is believed to provide tools for staging and typing different cancers (Shi et al., 2003; Beck et al., 1999).

DNA methylation patterns are created during development by a combination of methylation and demethylation events (Razin and Cedar, 1993). DNA methylation is catalyzed by DNA methyltransferases, which transfer a methyl group from the methyl donor *S*-adenosyl-L-methionine (SAM) onto the 5' of the cytosine ring (Wu and Santi, 1985). It was proposed that de novo methyltransferase enzymes, which do not discriminate between unmethylated and hemimethylated DNA, generate new DNA methylation patterns (Razin and Riggs, 1980). It has been a long-standing belief that these de novo methyltransferases are restricted to development and that once DNA methylation patterns are formed, they are fixed for life and faithfully copied by a maintenance DNA methyltransferase (Razin

* Tel.: +1-514-398-7107; fax: +1-514-398-6690.
E-mail address: mszyf@pharma.mcgill.ca (M. Szyf).

and Riggs, 1980). The maintenance enzyme is hypothesized to only methylate a hemimethylated substrate, which is generated when a DNA bearing a methylated CpG dinucleotide is newly replicated (Gruenbaum et al., 1982). Replication of a non-methylated CpG dinucleotide generates a double-stranded, non-methylated CpG dinucleotide pair, which is not a substrate for maintenance DNA methyltransferase. This ensures that the DNA methylation pattern is accurately replicated during cell division. DNA methyltransferase 1 (DNMT1) fits the description of a maintenance DNA methyltransferase (Bestor et al., 1988; Bacolla et al., 1999), whereas DNMT3a and 3b do not discriminate between non-methylated and hemimethylated substrates and thus fit the description of de novo methyltransferases (Hsieh, 1999; Okano et al., 1999). Genetic experiments with mice bearing *dnmt1*—/— knock-outs confirmed DNMT1's role as a primary DNA methyltransferase (Li et al., 1992) and the de novo role of DNMT3a and 3b were confirmed using *dnmt3a*—/— and *dnmt3b*—/— knock-outs (Okano et al., 1999).

However, several studies indicate that the differentiation between de novo and maintenance methylation is perhaps more blurred than originally thought. This has obvious important implications on our understanding of the changes in DNA methylation in cancer as will be discussed below.

- (1) It has been shown that DNMT3a and b are required for the maintenance of DNA methylation of certain sequences (Liang et al., 2002; Chen et al., 2003).
- (2) DNMT1 can catalyze de novo methylation in vitro and is stimulated by methylated CpGs in *cis*, thus it can lead to de novo spreading of DNA methylation from a methylated center (Bacolla et al., 1999).
- (3) DNMT3a and b are physically associated with DNMT1 suggesting that they might cooperate in maintenance and de novo methylation in somatic cells (Kim et al., 2002).
- (4) De novo methylation of specific sequences takes place in somatic cells and is not restricted to embryogenesis (Szyf et al., 1989).
- (5) Aberrant de novo methylation events occur not only in cancer but also in normal tissues and are evident during normal aging (Ahuja et al., 1998; Issa et al., 1996).
- (6) De novo methylation could be rapidly targeted to specific sequences in somatic cells by transacting factors such as the promyelocytic leukemia–retinoic acid receptor (PML–RAR) fusion protein (Di Croce et al., 2002) suggesting that methylation in somatic cells is not exclusively determined by the methylation state of the parental strand template.

These issues with the classic maintenance DNA methylation theory prompted us to propose an alternative hypothesis that DNA methylation patterns are dynamically maintained through life and are not determined exclusively by the methylation pattern of the template paternal strand as will be discussed below.

2. Demethylase and demethylation

A dynamic pattern of methylation requires the presence of both methylating and demethylating activities. Most known biological signals are indeed reversible. However, it was believed that the DNA methylation reaction is irreversible and that true removal of a methyl group from the methylated cytosine ring is highly unlikely. Therefore, DNA methylation was considered to be fixed and faithfully replicated through life post-development and irresponsive to the diverse conditions that an organism is exposed to throughout its mature life.

Although it was believed, as discussed above, that the only activity which maintains DNA methylation post-development is maintenance DNA methyltransferase, it was clear that multiple global and gene-specific demethylation events occur during development (Razin et al., 1984; Benvenisty et al., 1985; Cedar and Razin, 1990; Shemer et al., 1991; Kafri et al., 1992; Brandeis et al., 1993). What enzymatic activity is responsible for this? The first proposed mechanism was a passive mechanism; replication in the absence of DNA methyltransferase would result in non-methylated DNA (Razin and Riggs, 1980). One possibility of achieving this is by either masking specific sites from DNA methyltransferase by transacting factors at the time of DNA replication, or by a general reduction in DNA methyltransferase activity, which would result in global demethylation (Monk et al., 1991). Since it was demonstrated in several studies in vivo (Kafri et al., 1993; Oswald et al., 2000) and in vitro (Wilks et al., 1984; Szyf et al., 1985; Razin and Cedar, 1991; Paroush et al., 1990; Frank et al., 1991) that demethylation could occur in the absence of cell division, other mechanisms were sought such as glycosylase activities removing the methylated cytosine followed by repair in the absence of DNA methyltransferase of the abasic site (Jost, 1993; Jost et al., 1995; Razin et al., 1986). Another mechanism proposed that a nucleotide excision and replacement activity is responsible for exchanging the methylated CpG dinucleotide with a non-methylated version (Weiss et al., 1998). The main problem with the repair-based hypotheses is that they cannot explain the global demethylation occurring a few hours post replication in the absence of cell division (Oswald et al., 2000). If a global excision and repair of the genome has to take place to bring about global hypomethylation, this could seriously damage the integrity of the genome at an extremely critical point. We therefore proposed the presence of a true demethylase, which removes methyl groups without damaging the DNA and extracted a demethylase from human lung carcinoma cells (Ramchandani et al., 1999). We also cloned a demethylase cDNA, which was identical to methylated DNA binding protein 2 (*MBD2*) (Bhattacharya et al., 1999). The assignment of demethylase activity to *MBD2* is highly contested and several group were unable to confirm these results (Ng et al., 1999; Zhang et al., 1999; Boeke et al., 2000). Nevertheless, in recent experiments we show

that human embryonal kidney HEK 293 cells express a demethylase activity that targets methylated DNA when it is associated with acetylated histones (Cervoni et al., 2002; Cervoni and Szyl, 2001; Detich et al., 2002) and that this activity is inhibited in the cells when MBD2 is knocked down by expression of an antisense *MBD2* RNA (Detich et al., 2003a). Although the assignment of demethylase activity to *MBD2* is controversial, there are data to suggest that mammalian cells bear demethylase activity and that the DNA methylation reaction is a reversible biological signal similar to other biological signals such as acetylation, phosphorylation and glycosylation.

3. Site specificity of DNA methylation patterns

DNA methylation patterns exhibit site and tissue specificity; how are these determined? This question is extremely important in cancer where aberrant methylation events are documented. To understand how DNA methylation patterns are altered in cancer we must first formulate a working hypothesis on how this specificity is generated and maintained under normal conditions. According to the classic model DNA methylation patterns are specifically maintained because the maintenance methyltransferase accurately copies the methylation pattern from the paternal strand onto the daughter strand during cell division (Razin and Riggs, 1980). However, this hypothesis still leaves the question of how these patterns were generated in the first place

by site-specific de novo methylation during development unanswered.

4. Relationship between DNA methylation and chromatin

4.1. Inactive chromatin triggers de novo methylation

It is proposed here that the answer to the question of the specificity of DNA methylation pattern could come from revisiting the data demonstrating a relation between chromatin structure and DNA methylation. As discussed above, one of the earliest observation in vertebrate DNA methylation was the observation that methylated DNA is associated with inactive chromatin (Razin and Cedar, 1977). Later observations established that methylated DNA distinguishes regulatory regions of inactive genes. This relationship between chromatin and DNA methylation could be understood both ways, either that DNA methylation could cause inactive chromatin structure and silencing of gene expression or that inactive chromatin triggers DNA methylation (Fig. 1). The classic model was the former that DNA methylation precipitates an inactive chromatin structure (Eden et al., 1998; Hashimshony et al., 2003; Keshet et al., 1986). Molecular links between DNA methylation and chromatin structure are methylated DNA binding proteins such as MeCP2 (Meehan et al., 1992; Lewis et al., 1992). MeCP2, as well as other methylated DNA-binding proteins, recruit

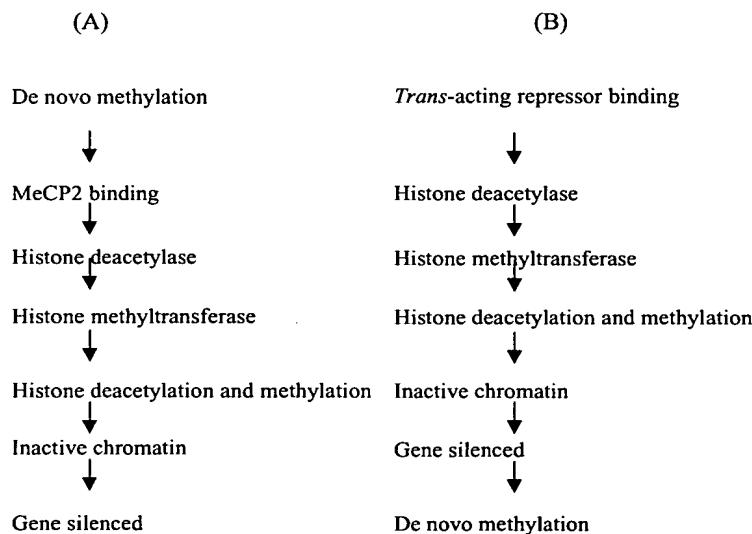


Fig. 1. Two alternative models of silencing and methylation of tumor suppressor genes in cancer. Model A: a tumor suppressor gene is de novo methylated. The mechanisms targeting de novo methyltransferase to specific tumor suppressor genes are unknown, high levels of DNA methyltransferases in cancer cells might enhance this process. DNA methylation leads to binding of MeCP2. MeCP2 recruits histone deacetylases and histone methyltransferases to the gene. Histone modification catalyzed by these enzymes leads to chromatin inactivation and silencing of the gene. Model B: a *trans*-acting repressor interacts with a tumor suppressor gene. This repressor recruits histone deacetylases and histone methyltransferases to the gene resulting in inactive chromatin. The histone deacetylase and the histone methyltransferase recruit DNA methyltransferase to the gene resulting in de novo methylation of the gene.

the co-repressor Sin3A, histone deacetylases (HDAC) and K9-histone methyltransferases to methylated DNA. These recruited enzymes modify the histone tails associated with methylated DNA resulting in a closed inactive configuration of chromatin (Nan et al., 1997; Ng et al., 1999, 2000; Bird and Wolffe, 1999; Ng and Bird, 1999; Fuks et al., 2003b) (Fig. 1A).

Recent data however supports the alternative hypothesis that inactive chromatin structure brings about increased DNA methylation (Ben-Porath and Cedar, 2001), whereas active chromatin leads to demethylation (Szyf, 2003). There is genetic evidence from *Neurospora* (Tamaru and Selker, 2001), plants (Jackson et al., 2002; Jeddeloh et al., 1999), mice (Dennis et al., 2001) (Lehnertz et al., 2003) and humans (Gibbons et al., 2000) that mutations in genes encoding chromatin remodeling and histone modification proteins also cause varying degrees of altered DNA methylation. The most interesting connection that is being currently unraveled is the association between K9-histone methyltransferases, heterochromatin protein 1(HP1)- a methylated histone binding protein and DNA methylation (Lehnertz et al., 2003; Jackson et al., 2002; Tamaru and Selker, 2001). One molecular explanation for this relation is that histone methyltransferase SUV39, HDACs and HP1 physically associate with DNMTs (Fuks et al., 2003a). It was therefore proposed that chromatin structure inactivation precedes DNA methylation and recruits DNMTs to inactive chromatin (Szyf, 2003) (Fig. 1B).

This relation between chromatin and DNA methylation could provide an explanation for both the association of inactive chromatin with methylated DNA and the site specificity of DNA methylation since it is known that site-specific repressors target histone deacetylases and histone methyltransferases to specific promoters. In accordance with this hypothesis it was recently shown that the tumor suppressor p16 is inactivated by chromatin structure modifications in the absence of DNA methyltransferases suggesting that the observed methylation of p16 in some cancer cells follows inactivation of chromatin (Bachman et al., 2003). A similar mechanism was proposed to target the methylation of *glutathione S-transferase P1* gene in prostate cancer (Song et al., 2002). If chromatin directs DNA methyltransferases both maintenance and de novo methylation could be explained by the same mechanism whereby new inactive chromatin results in de novo methylation and maintaining inactive chromatin structure results in maintenance DNA methylation. An excellent example of a chromatin repressor targeting DNA methyltransferases to target promoters is the leukemia-promoting PML–RAR fusion protein, which recruits histone deacetylases and DNA methyltransferase to genes bearing the cognate recognition sequence (Di Croce et al., 2002).

The hypothesis linking DNA methylation and chromatin structure also implies that as long as the chromatin structure is dynamic so is the DNA methylation pattern and it could potentially change at any point during life when there is a change in chromatin structure (Szyf, 2003). This hypothesis

has important implications on our understanding of aberrant regional methylation in cancer, its causes and its relative role in transformation. Regional hypermethylation is caused by regional changes in chromatin structure, which according to this hypothesis are the primary causes of tumor suppressor silencing in cancer (Fig. 2).

The data showing that chromatin directs DNA methylation has to be reconciled with data from transfection experiments showing that DNA methylation brings about inactive chromatin (Eden et al., 1998; Keshet et al., 1986; Hashimshony et al., 2003). In addition, methylated DNA binding proteins such as MeCP2 recruit histone modification proteins resulting in chromatin inactivation suggesting that DNA methylation is a cause of chromatin inactivation (Nan et al., 1998). One possible resolution of this question proposes that the role of DNA methylation is to guard inactive chromatin against an aberrant drift in the chromatin structure (Szyf, 2003). The association of inactive chromatin with methylated DNA assures that an aberrant loss of histone modification is immediately corrected by MeCP2 recruited histone methyltransferases and histone deacetylases. Alternatively both systems, DNA methylation and chromatin modification, have complementary roles in stabilizing gene silencing.

4.2. Active chromatin structure triggers DNA demethylation

If DNA methylation reflects the dynamic changes in chromatin then activation of chromatin should lead to DNA demethylation. In accordance with this hypothesis the HDAC inhibitor sodium butyrate triggers replication-independent, global hypomethylation in *Epstein Barr Virus* (*EBV*) transformed cells (Szyf et al., 1985) and trichostatin A (TSA) the HDAC inhibitor induces hypomethylation in *Neurospora* (Selker, 1998). Ectopically methylated DNA controlled by a strong promoter is actively demethylated in human HEK 293 cells, TSA induces demethylation (Cervoni and Szyf, 2001) and inhibitors of acetylation complexes (InHAT) inhibit active demethylation (Cervoni et al., 2002). Based on these data we suggested that the state of DNA methylation is an equilibrium of methyltransferase and demethylase reactions and the direction of the equilibrium is determined by chromatin structure (Szyf et al., 1985) (Fig. 2). This provides a simple explanation for the correlation of hypomethylated DNA, active chromatin and gene expression. The same mechanism could be responsible for demethylation of active genes during development, the maintenance of active genes in a hypomethylated state and the potential demethylation of genes later in life. In accordance with this hypothesis it was shown that the developmental demethylation of the κ *immunoglobulin* gene requires the intronic enhancer and the transacting factor NF κ B (Kistler et al., 1997; Kirillov et al., 1996; Ji et al., 2003).

One important implication of this model is that DNA methylation patterns could be altered even in postmitotic

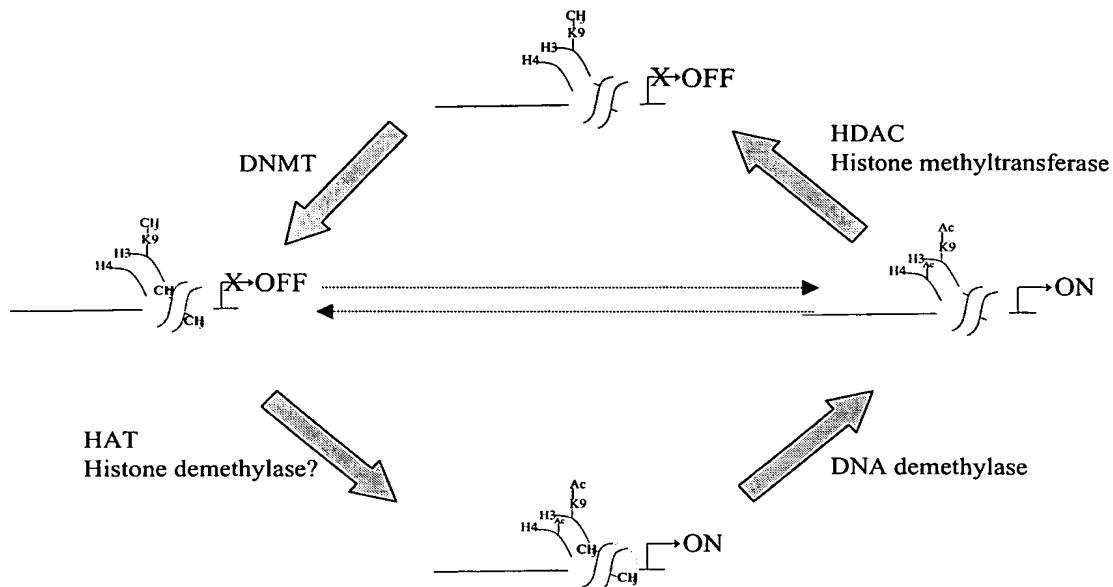


Fig. 2. DNA methylation is an equilibrium of methylation and demethylation, the direction of the reaction is determined by chromatin structure. A gene is found in either a methylated state, which is associated with inactive chromatin or an unmethylated state associated with active chromatin. Intermediate states are shown as well. One nucleosome is shown. CH₃, methyl group; Ac, acetyl group; K9, lysine 9; H3, histone 3 tail; H4, histone 4 tail. Demethylation of a gene is highly unlikely when it is associated with inactive chromatin and methylation is highly unlikely when the gene is associated with active chromatin. A conversion from a methylated state to a demethylated state requires a change in chromatin structure catalyzed by histone acetyltransferases HAT and a putative histone demethylase. Once chromatin is active, demethylase gains access to the gene and demethylates it. A gene found in an inactive chromatin structure remains methylated even when DNA demethylase is abundantly present. A conversion from a non-methylated to a methylated state requires first the conversion of the chromatin to an inactive state. This could be catalyzed by recruitment of histone deacetylases HDAC and histone methyltransferases to the gene by a *trans*-acting repressor. The HDAC and histone methyltransferase recruit DNMT (DNA methyltransferase) to the gene resulting in methylation of the gene. The presence of a repressor associated with a gene would maintain the gene in an inactive chromatin structure and protect it from demethylation.

tissues in the absence of cell division and that drugs that alter chromatin structure would also alter DNA methylation in postmitotic tissue. In accordance with this hypothesis we recently showed that valproate, an antiepileptic drug, which inhibits histone deacetylase, also triggers active DNA demethylation (Detich et al., 2003a). Similarly, if active demethylation plays a role in maintaining active genes unmethylated, then inhibitors of demethylase would cause hypermethylation. The methyl donor (SAM) was recently shown to inhibit demethylase activity and cause ectopic methylation by inhibiting demethylation of an ectopically methylated gene (Detich et al., 2003b). This also suggests that it might be possible to develop DNA replication-independent agents for modifying DNA methylation patterns.

5. Mechanisms of regional hypermethylation, global hypomethylation and aberrant DNA methyltransferase expression in cancer

The classic concept of fixed DNA methylation patterns and their inheritance could not explain the paradoxical DNA

methylation aberrations in cancer, the coexistence of global hypomethylation, increased DNA methyltransferase and regional hypermethylation. The dynamic relationship between DNA methylation and chromatin proposed here can provide a consistent rationalization for these observations. The epigenetic silencing of tumor suppressor genes by regional hypermethylation is well documented (Baylin et al., 1998, 2001). Regional hypermethylation is proposed here to be triggered by site-specific chromatin changes; these changes target DNA methylation to specific regions and are independent of the general levels of DNA methyltransferases and demethylase. Therefore, the extent of CpG island methylation does not correlate with the global cellular levels of DNA methyltransferases (Eads et al., 1999; Sato et al., 2002; Saito et al., 2001). Chromatin inactivation of tumor suppressors can occur in the absence of DNA methyltransferase as has recently been proposed (Bachman et al., 2003).

Although chromatin is proposed to be the primary cause of epigenetic silencing, DNA methyltransferase and DNA methylation obviously play an important role in maintenance of silencing of tumor suppressor genes since either knock-down of DNA methyltransferase (Fournel et al., 1999; Robert et al., 2003) or inhibition of DNA methylation

by 5-azacytidine (5-azaC) reactivate methylation-silenced tumor suppressor genes (Otterson et al., 1995). However we should be cautious about the interpretation of 5-azaC experiments. 5-azaC was shown to inhibit histone methylation as well as inhibit DNA demethylation (Nguyen et al., 2002). It remains unclear whether these are two independent activities of 5-azaC. If 5-azacytidine is also a histone methylation inhibitor then one can argue that activation of silenced tumor suppressor genes by 5-azacytidine is primarily caused by a change in chromatin structure and not DNA demethylation. Similarly DNA methyltransferases were shown to recruit histone deacetylases and histone methyltransferases (Fuks et al., 2000, 2003a). Knock-down of DNMT might be primarily acting by a DNA-methylation-independent mechanism as has been previously demonstrated (Milutinovic et al., 2000, 2003).

Another important observation in cancer is the high level of DNA methyltransferases reported (Issa et al., 1993). Although an attractive hypothesis is that this increase in DNA methyltransferase activity is responsible for the hypermethylation of CpG islands, it is now clear that there is no clear correlation between the high levels of DNA methyltransferase and CpG island methylation as discussed above. DNMT1 is a multifunctional protein that plays roles in DNA methylation

as well as in suppression of gene expression by a mechanism that is independent of its DNA methylation catalytic activity (Szyf et al., 2000; Szyf, 2001). DNMT1 associates with HDAC1 (Fuks et al., 2000) and HDAC2 (Rountree et al., 2000), the tumor suppressor Rb (Robertson et al., 2000), and the DNA replication protein proliferating cell nuclear antigen (PCNA) (Chuang et al., 1997). A different domain in the DNMT1 protein is responsible for each of these interactions. DNMT1 is also required for initiation of DNA replication most probably by a methylation-independent pathway (Knox et al., 2000; Milutinovic et al., 2003). Knock-down of DNMT1 with antisense oligonucleotides in human cancer lines leads to an intra-S phase arrest (Milutinovic et al., 2003). The disruption of cell cycle regulation of DNMT1 rather than its overall level were shown to be critical for cellular transformation (Detich et al., 2001). Taken together it is hypothesized that DNMT1 and possibly other DNMTs play a regulatory role in the cell cycle that is independent of DNA methylation (Fig. 3).

The third observation, which is a hallmark of cancer is the global hypomethylation which includes unique and repetitive sequences (Feinberg et al., 1988; Narayan et al., 1998; Qu et al., 1999a,b). The fact that hypomethylation is general suggests that the defect is in the general DNA methylation

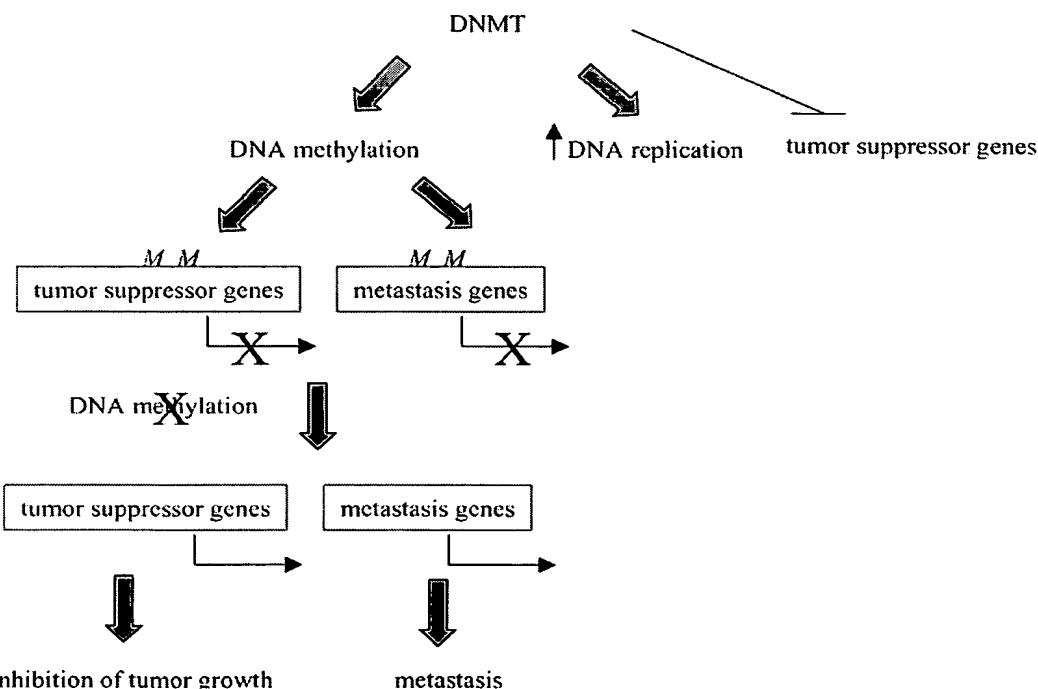


Fig. 3. DNMT1 roles in transformation, possible consequences of DNA methylation inhibition. DNMT is a multifunctional protein, which plays different roles in cellular transformation. DNMT catalyzes DNA methylation. DNA methylation suppresses tumor suppressor genes as well as genes required for metastasis. DNMT also plays a role in stimulating DNA replication and controls the expression of some tumor suppressor genes by a methylation-independent mechanism. Inhibition of the catalytic activity of DNMT will lead to demethylation and reexpression of tumor suppressor genes as well as metastatic genes. This might result in increased metastasis. Inhibiting the methylation-independent functions of DNMT might not result in demethylation and avoid the untoward effects of DNA methylation inhibition.

machinery and not in the interaction of unique sequences with some distinct factors. It is highly unlikely that the defect is in the DNA methyltransferase machinery since DNMTs are highly expressed in most cancers including tumors with a high degree of global hypomethylation (Jurgens et al., 1996). Therefore, it stands to reason that there is an increase in the DNA demethylation capacity in the cell. However, the protein(s) involved in demethylation in tumor cells remains unclear.

We have previously proposed that MBD2/demethylase is involved in DNA demethylation (Bhattacharya et al., 1999). This assignment of demethylase activity has been highly controversial as discussed above. MBD2 was proposed on the other hand to be a component of the MeCP1 methylated DNA binding complex and be involved in gene silencing rather than demethylation (Ng et al., 1999). There is conclusive data however that MBD2 plays a critical role in cancer. Antisense knock-down of MBD2 inhibits tumorigenesis of human cancer lines in vitro and in vivo (Slack et al., 2001). A recent study demonstrated that MBD2 is required for colorectal cancer in the min^{-/-} mouse model (Sansom et al., 2003). To resolve the question of whether MBD2 is involved in demethylation in tumors, the demethylase activities from tumors must be characterized.

What is the role of global hypomethylation in cancer? One possible mechanism is that hypomethylation destabilizes the genome, which might promote some of the chromosomal rearrangements seen in cancer (Gaudet et al., 2003; Eden et al., 2003; Tuck-Muller et al., 2000; Chen et al., 1998). Another possible mechanism is that hypomethylation triggers activation of genes, which are required for tumorigenesis. A candidate group of genes are pro-metastatic genes. A number of genes involved in metastasis were shown to be hypomethylated in metastatic cancers (Nakamura and Takenaga, 1998; Shen et al., 1998; Rosty et al., 2002; Ormerod et al., 1986). A good example is the protease *urokinase plasminogen activator* (*uPA*) which is required for tumor invasion and metastasis is expressed in highly metastatic breast cancer and is not expressed in non-metastatic breast cancer cell lines (Guo et al., 2002). Treatment of a non-metastatic cancer cell line MCF-7 with the DNA methylation inhibitor 5-azaC results in activation of *uPA* and induction of metastasis (Guo et al., 2002). It was proposed that hypomethylation is required for a later state of tumorigenesis when tumors move away from their original site whereas hypermethylation and increased DNMT activity is required for the first step of tumorigenesis when cells need to override the normal cell cycle regulatory mechanisms and shift into uninhibited growth mode (Guo et al., 2002). In accordance with this hypothesis it was shown that 5-azaC stimulates metastasis in cancer cell lines (Olsson and Forchhammer, 1984; Ormerod et al., 1986; Habets et al., 1990).

The model of regional hypermethylation proposed here is consistent with the persistence of hypermethylation in cells expressing high demethylation activity in cancer. We pro-

posed that the accessibility of a methylated gene to demethylase activity depends on its chromatin structure (Cervoni and Szyf, 2001). For example, the InHAT complex, which inhibits histone acetylation, also prohibits demethylation even in the presence of high demethylating activity in the cell (Cervoni et al., 2002). Thus, if regional hypermethylation involves local changes in chromatin structure that render it inactive as predicted, such a gene would not be accessible to demethylase. Indeed high levels of demethylase would not cause demethylation of genes whose chromatin is inactivated.

In summary, the model presented here which suggests that DNA methylation reaction is an equilibrium whose direction is dependent on chromatin structure is consistent with the principal hallmarks of DNA methylation in cancer.

6. DNA methylation and anticancer therapy

Inhibitors of DNMT1 were the first goal of anticancer therapy targeting DNA methylation (Szyf, 1994). The accepted objective of most of the current attempts at DNA methyltransferase inhibitors is to identify potent small-molecule inhibitors of DNA methyltransferase activity. The rationale behind this approach is to inhibit the high DNMT1 activity in tumors and to bring about demethylation and activation of tumor suppressor genes. There are two DNMT1 inhibitors currently in clinical trials: 5-aza-2'-deoxycytidine (DAC) and MG98, an antisense oligonucleotide directed at DNMT1 (Fournel et al., 1999).

6.1. Nucleoside analog inhibitors of DNA methyltransferase

The first group of DNA methylation inhibitors is the nucleoside analogs. The first drugs in this group are 5-azaC and DAC (Jones, 1985a,b). These pro-drugs are converted to their tri-nucleotide derivatives and act as inhibitors of DNA methyltransferase only after they are incorporated into DNA. DAC in DNA binds and traps the DNA methyltransferase in the replication fork, which proceeds in the absence of DNA methyltransferase, synthesizing DNA in the absence of methylation, resulting in passive demethylation (Jones and Taylor, 1981). The main concern about DAC is that its toxicity resembles other nucleoside analogues and that it might trap proteins other than DNMT1 resulting in effects independent of its effect on DNMT (Juttermann et al., 1994). 5-azaC is reported to have genetic effects, including mitotic recombination and point mutations, in the yeast *Saccharomyces cerevisiae* which does not bear DNA methylation, supporting concerns that 5-azaC has non-specific effects (Zimmermann and Scheel, 1984). An additional limitation of DAC is that it inhibits DNA methylation only in dividing cells and is ineffective in inhibiting DNMT in non-dividing tissues such as neurons in the brain, although they express significant amounts of DNMT (Goto et al., 1994; Deng and Szyf, 1998). Since DNA methylation is speculated to

be involved in central nervous system (CNS) pathologies such as schizophrenia (Chen et al., 2002; Tremolizzo et al., 2002) and fragile X syndrome (Chiurazzi et al., 1998), there is obviously a requirement for DNA methylation inhibitors which are independent of cell division.

DAC is nevertheless a very effective demethylating agent and activates methylation-silenced tumor suppressor genes quite effectively (Merlo et al., 1995). DAC also relieves other chromatin structure modifications such as histone methylation as discussed above (Nguyen et al., 2002). However, it remains unclear whether the effects on histone methylation are a consequence of its inhibition of DNA methylation, or are independent effects of DAC.

A number of clinical trials assessed the anticancer potential of 5-azacytidine since the 1970s in both soft and solid tissue cancers. Different clinical trials with DAC have been launched recently. All trials reported toxicities observed with other chemotherapeutic nucleoside analogs agents such as leucopenia (Lomen et al., 1975), myelosuppression and gastrointestinal toxicity (Shnider et al., 1976), granulocytopenia (Velez-Garcia et al., 1977), hematologic toxicity (Weiss et al., 1977), severe myelosuppression, diarrhea, and phlebitis (Gaynon and Baum, 1983).

Since DAC is the deoxy-derivative of 5-azaC, it is expected to be incorporated into DNA but not RNA and thus to be less toxic than 5-azaC. Preclinical tests of its toxicity in mice revealed the regular nucleoside analog toxicities such as bone marrow hypoplasia, necrosis of the small intestinal mucosa and atrophy of thymus and testes (Momparler and Frith, 1981). As far as clinical anti-tumor effects are concerned, previous trials showed good activity in leukemias but results in solid tumors were generally disappointing. The main challenge is to find a regimen that limits hematopoietic toxicity but maintains demethylation activity (Anonymous, 2002; Aparicio and Weber, 2002). Different trials and combinations, especially with chromatin modifiers such as histone deacetylase inhibitors, are underway.

The limitations of DAC implies that DNA methylation inhibitors which are not incorporated into DNA and are not dependent on cell division are needed to fully assess the therapeutic potential of DNA methylation inhibitors.

A different nucleoside analog recently shown to inhibit DNA methylation, induce tumor suppressor genes and inhibit tumorigenesis in mice with reduced toxicity and oral bioavailability is zebularine [1-(beta-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one] (Cheng et al., 2003). Although as a cytosine analog it is expected to have the general nucleoside analog side effects of DAC, its favorable pharmacokinetic properties make it a good candidate for a therapeutic DNA demethylating agent.

6.2. Non-nucleoside inhibitors of DNA methyltransferase

Other non-specific inhibitors of DNA methylation, which probably act at a different site than nucleoside analogues, are the 4-amino-benzoic acid derivatives procaine (Villar-Garea

et al., 2003) and procainamide (Cornacchia et al., 1988; Lin et al., 2001; Scheinbart et al., 1991). Procainamide and procaine are antiarrhythmic drugs and have been shown to induce global hypomethylation and demethylation of specific methylated CG islands as well as to inhibit growth of human cancer cell lines. The known side effect of procainamide, induction of lupus-like autoimmune disease, is thought to be brought about by demethylation of DNA in T cells, which lose the requirement for antigen and become autoreactive (Cornacchia et al., 1988). As non-nucleoside inhibitors these two drugs are not expected to exhibit the side effects observed with nucleoside analogues. The advantage of procainamide is that it has been used in therapy for a long time and its clinical pharmacology is well known. It is interesting to see whether future clinical trials would support an anti-cancer therapeutic potential for these drugs.

6.3. Concerns with the use of demethylating agents in cancer; stimulation of metastasis by demethylation

While most of the attention in DNA methylation-based anticancer therapy is directed toward demethylation and activation of tumor suppressor genes, there are data to suggest (as discussed above) that demethylation plays a causal role in cancer and specifically in metastasis (Szyf, 2003). Mice bearing hypomorphic *dnmt1* alleles resulting in wide genomic hypomethylation developed T cell lymphomas indicating a causal role for genomic hypomethylation in tumor formation (Gaudet et al., 2003). Although potent demethylating agents would inhibit tumor growth by activating tumor suppressor, there is a risk that they might simultaneously unleash pro-metastatic genes such as the reported induction of *uPA* in the non-metastatic breast cancer cell line MCF7 by DAC treatment (Guo et al., 2002). Although this risk does not necessarily imply that inhibitors of DNA methylation would be of no value, it suggests that more efforts should be invested in dissecting the differential roles of hyper- and hypo-methylation throughout cancer progression. It is possible that demethylating agents would only affect non-metastatic cancer. It stands to reason that in metastatic cancers the additional hypomethylation would have no effect on metastasis since pro-metastatic genes are hypomethylated prior to demethylation therapy. On the other hand, early stage non-metastatic, non-invasive cancer might be highly susceptible to the pro-metastatic effects of demethylating agents (Fig. 3).

An additional question that remains to be answered is whether all demethylating agents would demethylate the same repertoire of genes, or they would exhibit some gene specificity. It obviously would be interesting to test the possibility that sequence-specific agents could potentially be discovered. A putative mechanism for such agents might be targeting the associated factors required for directing DNMTs to specific genes and their silencing in cancer such as the leukemia-promoting PML-RAR fusion protein discussed above (Di Croce et al., 2002). Such an effort to discover

gene-specific demethylating agents will obviously require identifying the critical cofactors needed to target CG islands of tumor suppressor genes but not of metastatic genes. There is obviously an urgent need to gain more insight into the factors that target CpG islands such as p16 to chromatin inactivation and DNA methylation.

6.4. DNA methylation-independent DNMT inhibitors

One way to bypass the untoward effects of DNA demethylation in cancer is to target the methylation-independent functions of DNMT1 in cancer (Szyf et al., 2000; Milutinovic et al., 2000, 2003). DNMT1 has regulatory roles in DNA replication (Knox et al., 2000; Milutinovic et al., 2003) as well as gene silencing roles (Milutinovic et al., 2000) that are not mediated by its catalytic activity (Robertson et al., 2000). If one could knock-out these functions of DNMT1 and cause rapid, cell cycle arrest, passive demethylation, which requires DNA replication, could be inhibited (Milutinovic et al., 2003). One method to achieve this goal is to knockdown the DNMT1 protein rather than DNA methylation activity. An antisense oligonucleotides which knocks down DNMT1 was shown to induce a replication arrest and very limited DNA demethylation (Milutinovic et al., 2003). DNMT1 antisense oligonucleotides are now in clinical trials and it remains to be seen whether they would induce pro-metastatic genes. Also unknown is the extent of knock-down of DNMT1 that is required to arrest DNA replication and inhibit passive demethylation. Further structure-function studies of DNMT1 and cellular transformation are required in order to identify the domains in DNMT1 which are responsible for transformation in a DNA methylation-independent manner. These functions could become targets for small-molecule inhibitors in the future (Fig. 3).

6.5. Inhibiting global demethylation; prophylactic and therapeutic approaches

There is data to suggest that it is possible to control and protect against global hypomethylation by dietary means using methyl-enrichment diets (van der Westhuyzen, 1985; Ross, 2003). There is epidemiological data in humans that suggests that a combination of alcohol consumption and methyl deficient diet increases the risk of colon cancer (Giovannucci et al., 1995). One of the predicted mediators of these effects is the methyl donor SAM. Indeed it was shown in animal models that SAM can confer protection against chemically induced hepatocarcinogenesis (Pascale et al., 1995, 2002; Gerbracht et al., 1993) and that these effects are inhibited by the demethylating agent 5-aza-2'-deoxy-cytidine. It is believed that SAM acts by increasing the rate of DNA methylation reaction although there is no evidence as of yet that SAM concentration in the cell is limiting under any circumstance.

A new mechanism for SAM action recently proposed is that SAM increases DNA methylation by inhibiting

demethylase activity (Detich et al., 2003b). If, as proposed here, DNA methylation is a reversible reaction and a balance of methylation and demethylation reactions determines the DNA methylation pattern, then inhibition of demethylation should result in increased methylation. It is possible that SAM or its analogs would also inhibit the demethylase activity responsible for global hypomethylation in cancer. The main problem with using SAM as a therapy is the fact that SAM is highly unstable. It is therefore important to test the possibility that stable analogues of SAM could also inhibit demethylase activity and global demethylation in cancer. Another approach might be increasing SAM levels by enhancing methyl content in diet as discussed above (Ross, 2003) or by pharmacologically manipulating either SAM synthesis or metabolism. This class of drugs has yet to be discovered.

6.6. MBD2 inhibitors as anticancer agents

Methylated DNA-binding protein 2 (MBD2) was assigned two functions; a methylated DNA-binding protein which silences methylated genes (Hendrich and Bird, 1998; Ng et al., 1999) and a DNA demethylase (Bhattacharya et al., 1999). Irrespective of whether MBD2 is involved in tumor suppressor silencing or demethylation, there are data to suggest that it is a unique anticancer target. Inhibition of MBD2 by antisense knockdown inhibits tumorigenesis but does not inhibit cell growth and does not alter cell cycle kinetics demonstrating that MBD2 is not required for growth of either normal cells or transformed cells (Slack et al., 2002). Similarly, *mbd2*−/− knock-out mice are viable and fertile supporting the hypothesis that MBD2 is not required for cell growth (Hendrich et al., 2001), contrary to DNMT1 whose inhibition results in changes to the cell cycle (Knox et al., 2000, Milutinovic et al., 2003). Recent data demonstrated that *mbd2*−/− knock-out suppresses colorectal cancer in the *min*−/− mouse (Sansom et al., 2003). These data suggest that MBD2 is a unique anticancer target which is required for tumorigenesis but not for normal cell growth. Which genes are regulated by MBD2 is an open question. Presently there are no known inhibitors of MBD2. We have recently developed antisense inhibitors of MBD2, which were demonstrated to inhibit tumorigenesis of human tumor xenographs in mice *in vivo* (unpublished data). SAM was also shown to inhibit the demethylase activity of recombinant MBD2 (Detich et al., 2003b). There is obviously a great need to discover small-molecule inhibitors of MBD2 and test them as anticancer agents.

6.7. Replication-independent demethylating agents

As discussed above, nucleoside analogues and other potential inhibitors of DNA methyltransferase catalytic activity cause demethylation passively by inhibiting DNA methylation during replication. This limits their use to dividing cells, which excludes most of the postmitotic tissues

including the brain. Our understanding of the dynamic relation between chromatin structure and DNA methylation has opened up new possibilities to bring about demethylation by altering chromatin structure. A good example of such a drug is valproate, which has been used for some time as an antiepileptic and mood-stabilizing agent and was recently shown to promote replication-independent DNA demethylation by inhibiting histone deacetylases and increasing histone acetylation (Detich et al., 2003a). Inhibitors of histone methyltransferases might also be candidates for such an activity by activating the chromatin structure and increasing the accessibility to demethylase. It is believed that chromatin structure-modifying drugs that target histone-modifying enzymes would be developed in the coming years. These will be used for transient activation of chromatin by altering chromatin structure as well as stably activating genes by changing DNA methylation patterns. However, these agents might also induce pro-metastatic genes and should be therefore handled with care.

7. Summary and future directions

The DNA methylation machinery has a complex and seemingly paradoxical involvement in cancer. Regional hypermethylation coexists with global hypomethylation. This review proposes that this paradox could be partially explained by a hypothesis postulating that DNA methylation is a dynamic equilibrium of methylation and demethylation reactions and that the direction of this equilibrium is determined by chromatin structure. Local chromatin inactivation could explain regional hypermethylation while a global increase in demethylation capacity can explain global hypomethylation. DNA demethylating agents are potential anti-tumor agents since they lead to growth inhibition by bringing about passive demethylation and activation of tumor suppressor genes. Such agents might also exhibit a pro-metastatic effect by demethylating and activating genes involved in metastasis. One challenge is developing inhibitors of DNMT1 that target the growth suppressor effects without activating pro-metastatic genes. Another possibility is to develop DNMT1 inhibitors which cause arrest of DNA replication without causing demethylation. Hypomethylation is emerging as an important player in tumorigenesis and both dietary and pharmacological strategies need to be developed to inhibit global hypomethylation in cancer. The relationship between chromatin and DNA methylation provides us with new routes to design drugs that inhibit methylation without the requirement for cell division. The DNA methylation—putative demethylation—machinery provides us with multiple targets for anti-cancer therapy.

Acknowledgements

The work from our laboratory described in this review was funded by the national Cancer Institute of Canada and

the Canadian Institutes for Health Research. Thanks to Ian Weaver for his critical review of the manuscript and his thoughtful comments.

References

- Ahuja, N., Li, Q., Mohan, A.L., Baylin, S.B., Issa, J.P., 1998. Aging and DNA methylation in colorectal mucosa and cancer. *Cancer Res.* 58, 5489–5494.
- Anonymus, 2002. Decitabine has promising clinical activity in chronic myeloid leukemia. *Expert. Rev. Anticancer Ther.* 2, 620–621.
- Aparicio, A., Weber, J.S., 2002. Review of the clinical experience with 5-azacytidine and 5-aza-2'-deoxycytidine in solid tumors. *Curr. Opin. Investig. Drugs* 3, 627–633.
- Bachman, K.E., Park, B.H., Rhie, I., Rajagopalan, H., Herman, J.G., Baylin, S.B., Kinzler, K.W., Vogelstein, B., 2003. Histone modifications and silencing prior to DNA methylation of a tumor suppressor gene. *Cancer Cell.* 3, 89–95.
- Bacolla, A., Pradhan, S., Roberts, R.J., Wells, R.D., 1999. Recombinant human DNA (cytosine-5) methyltransferase. II. Steady-state kinetics reveal allosteric activation by methylated dna. *J. Biol. Chem.* 274, 33011–33019.
- Baylin, S.B., Herman, J.G., Graff, J.R., Vertino, P.M., Issa, J.P., 1998. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv. Cancer Res.* 72, 141–196.
- Baylin, S.B., Esteller, M., Rountree, M.R., Bachman, K.E., Schuebel, K., Herman, J.G., 2001. Aberrant patterns of DNA methylation chromatin formation and gene expression in cancer. *Hum. Mol. Genet.* 10, 687–692.
- Beck, S., Olek, A., Walter, J., 1999. From genomics to epigenomics: a loftier view of life. *Nat. Biotechnol.* 17, 1144.
- Ben-Porath, I., Cedar, H., 2001. Epigenetic crosstalk. *Mol. Cell* 8, 933–935.
- Benvenisty, N., Mencher, D., Meyuhas, O., Razin, A., Reshef, L., 1985. Sequential changes in DNA methylation patterns of the rat phosphoenolpyruvate carboxykinase gene during development. *Proc. Natl. Acad. Sci. U.S.A.* 82, 267–271.
- Bestor, T., Laudano, A., Mattaliano, R., Ingram, V., 1988. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J. Mol. Biol.* 203, 971–983.
- Bhattacharya, S.K., Ramchandani, S., Cervoni, N., Szyf, M., 1999. A mammalian protein with specific demethylase activity for mCpG DNA. *Nature* 397, 579–583.
- Bird, A.P., Wolffe, A.P., 1999. Methylation-induced repression—belts, braces, and chromatin. *Cell* 99, 451–454.
- Boeke, J., Ammerpohl, O., Kegel, S., Moehren, U., Renkawitz, R., 2000. The minimal repression domain of MBD2b overlaps with the methyl-CpG binding domain and binds directly to Sin3A. *J. Biol. Chem.* 275, 34963–34967.
- Brandeis, M., Ariel, M., Cedar, H., 1993. Dynamics of DNA methylation during development. *Bioessays* 15, 709–713.
- Cedar, H., Razin, A., 1990. DNA methylation and development. *Biochim. Biophys. Acta* 1049, 1–8.
- Cervoni, N., Szyf, M., 2001. Demethylase activity is directed by histone acetylation. *J. Biol. Chem.* 276, 40778–40787.
- Cervoni, N., Detich, N., Seo, S.B., Chakravarti, D., Szyf, M., 2002. The oncprotein Set/TAF-Ibeta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. *J. Biol. Chem.* 277, 25026–25031.
- Chen, R.Z., Pettersson, U., Beard, C., Jackson-Grusby, L., Jaenisch, R., 1998. DNA hypomethylation leads to elevated mutation rates. *Nature* 395, 89–93.

Chen, Y., Sharma, R.P., Costa, R.H., Costa, E., Grayson, D.R., 2002. On the epigenetic regulation of the human reelin promoter. *Nucleic Acids Res.* 30, 2930–2939.

Chen, T., Ueda, Y., Dodge, J.E., Wang, Z., Li, E., 2003. Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol. Cell Biol.* 23, 5594–5605.

Cheng, J.C., Matsen, C.B., Gonzales, F.A., et al., 2003. Inhibition of DNA methylation and reactivation of silenced genes by zebularine. *J. Natl. Cancer Inst.* 95, 399–409.

Chiurazzi, P., Pomponi, M.G., Willemsen, R., Oostra, B.A., Neri, G., 1998. In vitro reactivation of the FMR1 gene involved in fragile X syndrome. *Hum. Mol. Genet.* 7, 109–113.

Chuang, L.S., Ian, H.I., Koh, T.W., Ng, H.H., Xu, G., Li, B.F., 1997. Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for p21WAF1. *Science* 277, 1996–2000.

Cornacchia, E., Golbus, J., Maybaum, J., Strahler, J., Hanash, S., Richardson, B., 1988. Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *J. Immunol.* 140, 2197–2200.

Deng, J., Szyl, M., 1998. Multiple isoforms of DNA methyltransferase are encoded by the vertebrate cytosine DNA methyltransferase gene. *J. Biol. Chem.* 273, 22869–22872.

Dennis, K., Fan, T., Geiman, T., Yan, Q., Muegge, K., 2001. *Lsh*, a member of the *SNF2* family, is required for genome-wide methylation. *Genes Dev.* 15, 2940–2944.

Detich, N., Ramchandani, S., Szyl, M., 2001. A conserved 3'-untranslated element mediates growth regulation of *dna methyltransferase 1* and inhibits its transforming activity. *J. Biol. Chem.* 276, 24881–24890.

Detich, N., Theberge, J., Szyl, M., 2002. Promoter-specific activation and demethylation by MBD2/demethylase. *J. Biol. Chem.* 277, 35791–35794.

Detich, N., Bovenzi, V., Szyl, M., 2003a. Valproate induces replication-independent active DNA demethylation. *J. Biol. Chem.* 278, 27586–27592.

Detich, N., Hamm, S., Just, G., Knox, J.D., Szyl, M., 2003b. The methyl donor *S*-adenosylmethionine inhibits active demethylation of DNA: a candidate novel mechanism for the pharmacological effects of *S*-adenosylmethionine. *J. Biol. Chem.* 278, 20812–20820.

Di Croce, L., Rakcer, V.A., Corsaro, M., et al., 2002. Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science* 295, 1079–1082.

Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Danenberg, P.V., Laird, P.W., 1999. CpG island hypermethylation in human colorectal tumors is not associated with DNA methyltransferase overexpression [published erratum appears in *Cancer Res.* 59 (1999) 5860]. *Cancer Res.* 59, 2302–2306.

Eden, S., Hashimshony, T., Keshet, I., Cedar, H., Thorne, A.W., 1998. DNA methylation models histone acetylation. *Nature* 394, 842.

Eden, A., Gaudet, F., Waghmare, A., Jaenisch, R., 2003. Chromosomal instability and tumors promoted by DNA hypomethylation. *Science* 300, 455.

Feinberg, A.P., Gehrke, C.W., Kuo, K.C., Ehrlich, M., 1988. Reduced genomic 5-methylcytosine content in human colonic neoplasia. *Cancer Res.* 48, 1159–1161.

Fournel, M., Sapicha, P., Beaulieu, N., Besterman, J.M., MacLeod, A.R., 1999. Down-regulation of human DNA-(cytosine-5) methyltransferase induces cell cycle regulators p16(ink4A) and p21(WAF/Cip1) by distinct mechanisms. *J. Biol. Chem.* 274, 24250–24256.

Frank, D., Keshet, I., Shani, M., Levine, A., Razin, A., Cedar, H., 1991. Demethylation of CpG islands in embryonic cells. *Nature* 351, 239–241.

Fuks, F., Burgers, W.A., Brichm, A., Hughes-Davies, L., Kouzarides, T., 2000. DNA methyltransferase Dnmt1 associates with histone deacetylase activity. *Nat. Genet.* 24, 88–91.

Fuks, F., Hurd, P.J., Deplus, R., Kouzarides, T., 2003a. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. *Nucleic Acids Res.* 31, 2305–2312.

Fuks, F., Hurd, P.J., Wolf, D., Nan, X., Bird, A.P., Kouzarides, T., 2003b. The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J. Biol. Chem.* 278, 4035–4040.

Gaudet, F., Hodgson, J.G., Eden, A., et al., 2003. Induction of tumors in mice by genomic hypomethylation. *Science* 300, 489–492.

Gaynon, P.S., Baum, E.S., 1983. Continuous infusion of 5-azacytidine as induction for acute nonlymphocytic leukemia in patients with previous exposure to 5-azacytidine. *Oncology* 40, 192–194.

Gerbracht, U., Eigenbrodt, E., Simile, M.M., et al., 1993. Effect of *S*-adenosyl-L-methionine on the development of preneoplastic foci and the activity of some carbohydrate metabolizing enzymes in the liver, during experimental hepatocarcinogenesis. *Anticancer Res.* 13, 1965–1972.

Gibbons, R.J., McDowell, T.L., Raman, S., O'Rourke, D.M., Garrick, D., Ayyub, H., Higgs, D.R., 2000. Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nat. Genet.* 24, 368–371.

Giovannucci, E., Rimm, E.B., Ascherio, A., Stampfer, M.J., Colditz, G.A., Willett, W.C., 1995. Alcohol, low-methionine-low-folate diets, and risk of colon in men. *J. Natl. Cancer Inst.* 87, 265–273.

Goto, K., Numata, M., Komura, J.I., Ono, T., Bestor, T.H., Kondo, H., 1994. Expression of DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation* 56, 39–44.

Gruenbaum, Y., Cedar, H., Razin, A., 1982. Substrate and sequence specificity of a eukaryotic DNA methylase. *Nature* 295, 620–622.

Guo, Y., Pakneshan, P., Gladu, J., Slack, A., Szyl, M., Rabbani, S.A., 2002. Regulation of DNA methylation in human breast cancer. Effect on the *urokinase-type plasminogen activator* gene production and tumor invasion. *J. Biol. Chem.* 277, 41571–41579.

Habets, G.G., van der Kammen, R.A., Scholtes, E.H., Collard, J.G., 1990. Induction of invasive and metastatic potential in mouse T-lymphoma cells (BW5147) by treatment with 5-azacytidine. *Clin. Exp. Metastasis* 8, 567–577.

Hashimshony, T., Zhang, J., Keshet, I., Bustin, M., Cedar, H., 2003. The role of DNA methylation in setting up chromatin structure during development. *Nat. Genet.* 34, 187–192.

Hendrich, B., Bird, A., 1998. Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol. Cell. Biol.* 18, 6538–6547.

Hendrich, B., Guy, J., Ramsahoye, B., Wilson, V.A., Bird, A., 2001. Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. *Genes Dev.* 15, 710–723.

Hsieh, C.L., 1999. In vivo activity of murine de novo methyltransferases, Dnmt3a and Dnmt3b. *Mol. Cell. Biol.* 19, 8211–8218.

Issa, J.P., Vertino, P.M., Boehm, C.D., Newsham, I.F., Baylin, S.B., 1996. Switch from monoallelic to biallelic human *IGF2* promoter methylation during aging and carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.* 93, 11757–11762.

Issa, J.P., Vertino, P.M., Wu, J., et al., 1993. Increased cytosine DNA-methyltransferase activity during colon cancer progression. *J. Natl. Cancer Inst.* 85, 1235–1240.

Jackson, J.P., Lindroth, A.M., Cao, X., Jacobsen, S.E., 2002. Control of CpNgC DNA methylation by the kryptonite histone H3 methyltransferase. *Nature* 416, 556–560.

Jeddeloh, J.A., Stokes, T.L., Richards, E.J., 1999. Maintenance of genomic methylation requires a SWI2/SNF2-like protein. *Nat. Genet.* 22, 94–97.

Ji, Y., Zhang, J., Lee, A.I., Cedar, H., Bergman, Y., 2003. A multistep mechanism for the activation of rearrangement in the immune system. *Proc. Natl. Acad. Sci. U.S.A.* 100, 7557–7562.

Jones, P.A., 1985a. Altering gene expression with 5-azacytidine. *Cell* 40, 485–486.

Jones, P.A., 1985b. Effects of 5-azacytidine and its 2'-deoxyderivative on cell differentiation and DNA methylation. *Pharmacol. Ther.* 28, 17–27.

Jones, P.A., Taylor, S.M., 1981. Hemimethylated duplex DNAs prepared from 5-azacytidine-treated cells. *Nucleic Acids Res.* 9, 2933–2947.

Jost, J.P., 1993. Nuclear extracts of chicken embryos promote an active demethylation of DNA by excision repair of 5-methyldeoxycytidine. *Proc. Natl. Acad. Sci. U.S.A.* 90, 4684–4688.

Jost, J.P., Siegmann, M., Sun, L., Leung, R., 1995. Mechanisms of DNA demethylation in chicken embryos. Purification and properties of a 5-methylcytosine-DNA glycosylase. *J. Biol. Chem.* 270, 9734–9739.

Jurgens, B., Schmitz-Drager, B.J., Schulz, W.A., 1996. Hypomethylation of *L1 LINE* sequences prevailing in human urothelial carcinoma. *Cancer Res.* 56, 5698–5703.

Juttermann, R., Li, E., Jaenisch, R., 1994. Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11797–11801.

Kafri, T., Arieli, M., Brandis, M., et al., 1992. Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. *Genes Dev.* 6, 705–714.

Kafri, T., Gao, X., Razin, A., 1993. Mechanistic aspects of genome-wide demethylation in the preimplantation mouse embryo. *Proc. Natl. Acad. Sci. U.S.A.* 90, 10558–10562.

Keshtet, I., Lieman-Hurwitz, J., Cedar, H., 1986. DNA methylation affects the formation of active chromatin. *Cell* 44, 535–543.

Kim, G.D., Ni, J., Kelcoglu, N., Roberts, R.J., Pradhan, S., 2002. Co-operation and communication between the human maintenance and de novo DNA (cytosine-5) methyltransferases. *EMBO J.* 21, 4183–4195.

Kirillov, A., Kistler, B., Mostoslavsky, R., Cedar, H., Wirth, T., Bergman, Y., 1996. A role for nuclear NF-kappaB in B-cell-specific demethylation of the Igkappa locus. *Nat. Genet.* 13, 435–441.

Kistler, B., Baumann, B., Bergman, Y., Wirth, T., 1997. RelB is a key player for both kappa B-dependent transcription and demethylation in B cells. *Immunobiology* 198, 24–34.

Knox, J.D., Araujo, F.D., Bigey, P., Slack, A.D., Price, G.B., Zannis-Hadjopoulos, M., Szyf, M., 2000. Inhibition of DNA methyltransferase inhibits DNA replication. *J. Biol. Chem.* 275, 17986–17990.

Lehnertz, B., Ueda, Y., Derijck, A.A., et al., 2003. Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin. *Curr. Biol.* 13, 1192–1200.

Lewis, J.D., Meehan, R.R., Henzel, W.J., et al., 1992. Purification, sequence, and cellular localization of a novel chromosomal protein that binds to methylated DNA. *Cell* 69, 905–914.

Li, E., Bestor, T.H., Jaenisch, R., 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69, 915–926.

Liang, G., Chan, M.F., Tomigahara, Y., et al., 2002. Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. *Mol. Cell. Biol.* 22, 480–491.

Lin, X., Asgari, K., Putzi, M.J., et al., 2001. Reversal of *GSTP1* CpG island hypermethylation and reactivation of pi-class glutathione S-transferase (*GSTP1*) expression in human prostate cancer cells by treatment with procainamide. *Cancer Res.* 61, 8611–8616.

Lomen, P.L., Baker, L.H., Neil, G.L., Samson, M.K., 1975. Phase I study of 5-azacytidine (NSC-102816) using 24-h continuous infusion for 5 days. *Cancer Chemother. Rep.* 59, 1123–1126.

Mechan, R.R., Lewis, J.D., Bird, A.P., 1992. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucleic Acids Res.* 20, 5085–5092.

Merlo, A., Herman, J.G., Mao, L., et al., 1995. 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor *p16/CDKN2/MTS1* in human cancers. *Nat. Med.* 1, 686–692.

Milutinovic, S., Knox, J.D., Szyf, M., 2000. DNA methyltransferase inhibition induces the transcription of the tumor suppressor *p21/WAF1/CIP1/sdi1*. *J. Biol. Chem.* 275, 6353–6359.

Milutinovic, S., Zhuang, Q., Niveleau, A., Szyf, M., 2003. Epigenomic stress response. Knockdown of DNA methyltransferase 1 triggers an intra-S-phase arrest of DNA replication and induction of stress response genes. *J. Biol. Chem.* 278, 14985–14995.

Momparler, R.L., Frith, C.H., 1981. Toxicology in mice of the antileukemic agent 5-aza-2'-deoxycytidine. *Drug Chem. Toxicol.* 4, 373–381.

Monk, M., Adams, R.L., Rinaldi, A., 1991. Decrease in DNA methylase activity during preimplantation development in the mouse. *Development* 112, 189–192.

Nakamura, N., Takenaga, K., 1998. Hypomethylation of the metastasis-associated S100A4 gene correlates with gene activation in human colon adenocarcinoma cell lines. *Clin. Exp. Metastasis* 16, 471–479.

Nan, X., Campoy, F.J., Bird, A., 1997. MeCP2 is a transcriptional repressor with abundant binding sites in genomic chromatin. *Cell* 88, 471–481.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., Bird, A., 1998. Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature* 393, 386–389.

Narayan, A., Ji, W., Zhang, X.Y., et al., 1998. Hypomethylation of pericentromeric DNA in breast adenocarcinomas. *Int. J. Cancer* 77, 833–838.

Ng, H.H., Bird, A., 1999. DNA methylation and chromatin modification. *Curr. Opin. Genet. Dev.* 9, 158–163.

Ng, H.H., Zhang, Y., Hendrich, B., et al., 1999. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. *Nat. Genet.* 23, 58–61.

Ng, H.H., Jeppesen, P., Bird, A., 2000. Active repression of methylated genes by the chromosomal protein MBD1. *Mol. Cell. Biol.* 20, 1394–1406.

Nguyen, C.T., Weisenberger, D.J., Velicescu, M., et al., 2002. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. *Cancer Res.* 62, 6456–6461.

Okano, M., Bell, D.W., Haber, D.A., Li, E., 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 99, 247–257.

Olsson, L., Forchhammer, J., 1984. Induction of the metastatic phenotype in a mouse tumor model by 5-azacytidine, and characterization of an antigen associated with metastatic activity. *Proc. Natl. Acad. Sci. U.S.A.* 81, 3389–3393.

Ormerod, E.J., Everett, C.A., Hart, I.R., 1986. Enhanced experimental metastatic capacity of a human tumor line following treatment with 5-azacytidine. *Cancer Res.* 46, 884–890.

Oswald, J., Engemann, S., Lane, N., et al., 2000. Active demethylation of the paternal genome in the mouse zygote. *Curr. Biol.* 10, 475–478.

Otterson, G.A., Khleif, S.N., Chen, W., Coxon, A.B., Kaye, F.J., 1995. CDKN2 gene silencing in lung cancer by DNA hypermethylation and kinetics of p16INK4 protein induction by 5-aza 2'deoxyctydine. *Oncogene* 11, 1211–1216.

Paroush, Z., Keshtet, I., Yisraeli, J., Cedar, H., 1990. Dynamics of demethylation and activation of the alpha-actin gene in myoblasts. *Cell* 63, 1229–1237.

Pascale, R.M., Simile, M.M., De Miglio, M.R., et al., 1995. Chemoprevention by S-adenosyl-L-methionine of rat liver carcinogenesis initiated by 1,2-dimethylhydrazine and promoted by orotic acid. *Carcinogenesis* 16, 427–430.

Pascale, R.M., Simile, M.M., De Miglio, M.R., Feo, F., 2002. Chemoprevention of hepatocarcinogenesis: S-adenosyl-L-methionine. *Alcohol* 27, 193–198.

Qu, G., Dubeau, L., Narayan, A., Yu, M.C., Ehrlich, M., 1999a. Satellite DNA hypomethylation vs. overall genomic hypomethylation in ovarian epithelial tumors of different malignant potential. *Mutat. Res.* 423, 91–101.

Qu, G.Z., Grundy, P.E., Narayan, A., Ehrlich, M., 1999b. Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. *Cancer Genet. Cytogenet.* 109, 34–39.

Ramchandani, S., Bhattacharya, S.K., Cervoni, N., Szyf, M., 1999. DNA methylation is a reversible biological signal. *Proc. Natl. Acad. Sci. U.S.A.* 96, 6107–6112.

Razin, A., 1998. CpG methylation, chromatin structure and gene silencing—a three-way connection. *EMBO J.* 17, 4905–4908.

Razin, A., Cedar, H., 1977. Distribution of 5-methylcytosine in chromatin. *Proc. Natl. Acad. Sci. U.S.A.* 74, 2725–2728.

Razin, A., Cedar, H., 1991. DNA methylation and gene expression. *Microbiol. Rev.* 55, 451–458.

Razin, A., Cedar, H., 1993. DNA methylation and embryogenesis. *EXS* 64, 343–357.

Razin, A., Riggs, A.D., 1980. DNA methylation and gene function. *Science* 210, 604–610.

Razin, A., Shemer, R., 1999. Epigenetic control of gene expression. *Results Probl. Cell Differ.* 25, 189–204.

Razin, A., Szyl, M., 1984. DNA methylation patterns. Formation and function. *Biochim. Biophys. Acta* 782, 331–342.

Razin, A., Webb, C., Szyl, M., et al., 1984. Variations in DNA methylation during mouse cell differentiation in vivo and in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 81, 2275–2279.

Razin, A., Szyl, M., Kafri, T., et al., 1986. Replacement of 5-methylcytosine by cytosine: a possible mechanism for transient DNA demethylation during differentiation. *Proc. Natl. Acad. Sci. U.S.A.* 83, 2827–2831.

Robert, M.F., Morin, S., Beaulieu, N., et al., 2003. DNMT1 is required to maintain CpG methylation and aberrant gene silencing in human cancer cells. *Nat. Genet.* 33, 61–65.

Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L., Wolffe, A.P., 2000. DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. *Nat. Genet.* 25, 338–342.

Ross, S.A., 2003. Diet and DNA methylation interactions in cancer prevention. *Ann. N. Y. Acad. Sci.* 983, 197–207.

Rosty, C., Ueki, T., Argani, P., Jansen, M., Yeo, C.J., Cameron, J.L., Hruban, R.H., Goggins, M., 2002. Overexpression of *S100A4* in pancreatic ductal adenocarcinomas is associated with poor differentiation and DNA hypomethylation. *Am. J. Pathol.* 160, 45–50.

Rountree, M.R., Bachman, K.E., Baylin, S.B., 2000. DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci. *Nat. Genet.* 25, 269–277.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., Hirohashi, S., 2001. Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. *Hepatology* 33, 561–568.

Sansom, O.J., Berger, J., Bishop, S.M., Hendrich, B., Bird, A., Clarke, A.R., 2003. Deficiency of Mbd2 suppresses intestinal tumorigenesis. *Nat. Genet.* 34, 145–147.

Sato, M., Horio, Y., Sekido, Y., Minna, J.D., Shimokata, K., Hasegawa, Y., 2002. The expression of DNA methyltransferases and methyl-CpG-binding proteins is not associated with the methylation status of *p14(ARF)* *p16(INK4a)* and *RASSF1A* in human lung cancer cell lines. *Oncogene* 21, 4822–4829.

Scheinbart, L.S., Johnson, M.A., Gross, L.A., Edelstein, S.R., Richardson, B.C., 1991. Procainamide inhibits DNA methyltransferase in a human T cell line. *J. Rheumatol.* 18, 530–534.

Selker, E.U., 1998. Trichostatin A causes selective loss of DNA methylation in *Neurospora*. *Proc. Natl. Acad. Sci. U.S.A.* 95, 9430–9435.

Shemer, R., Eisenberg, S., Breslow, J.L., Razin, A., 1991. Methylation patterns of the human apoA-I/C-III/A-IV gene cluster in adult and embryonic tissues suggest dynamic changes in methylation during development. *J. Biol. Chem.* 266, 23676–23681.

Shen, L., Fang, J., Qiu, D., Zhang, T., Yang, J., Chen, S., Xiao, S., 1998. Correlation between DNA methylation and pathological changes in human hepatocellular carcinoma. *Hepatogastroenterology* 45, 1753–1759.

Shi, H., Maier, S., Nimmrich, I., Yan, P.S., Caldwell, C.W., Olek, A., Huang, T.H., 2003. Oligonucleotide-based microarray for DNA methylation analysis: principles and applications. *J. Cell. Biochem.* 88, 138–143.

Shnider, B.I., Baig, M., Colsky, J., 1976. A phase I study of 5-azacytidine (NSC-102816). *J. Clin. Pharmacol.* 16, 205–212.

Song, J.Z., Stirzaker, C., Harrison, J., Melki, J.R., Clark, S.J., 2002. Hypermethylation trigger of the *glutathione-S-transferase* gene (*GSTM1*) in prostate cancer cells. *Oncogene* 21, 1048–1061.

Szyl, M., 1994. DNA methylation properties: consequences for pharmacology. *Trends Pharmacol. Sci.* 15, 233–238.

Szyl, M., 2001. Towards a pharmacology of DNA methylation. *Trends Pharmacol. Sci.* 22, 350–354.

Szyl, M., 2003. Targeting DNA methylation in cancer. *Ageing Res. Rev.* 2, 299–328.

Szyl, M., Eliasson, L., Mann, V., Klein, G., Razin, A., 1985. Cellular and viral DNA hypomethylation associated with induction of Epstein-Barr virus lytic cycle. *Proc. Natl. Acad. Sci. U.S.A.* 82, 8090–8094.

Szyl, M., Schimmer, B.P., Seidman, J.G., 1989. Nucleotide-sequence-specific de novo methylation in a somatic murine cell line. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6853–6857.

Szyl, M., Knox, D.J., Milutinovic, S., Slack, A.D., Araujo, F.D., 2000. How does DNA methyltransferase cause oncogenic transformation? *Ann. N. Y. Acad. Sci.* 910, 156–174.

Tamaru, H., Selker, E.U., 2001. A histone H3 methyltransferase controls DNA methylation in *Neurospora crassa*. *Nature* 414, 277–283.

Tremolizzo, L., Carboni, G., Ruzicka, W.B., et al., 2002. An epigenetic mouse model for molecular and behavioral neuropathologies related to schizophrenia vulnerability. *Proc. Natl. Acad. Sci. U.S.A.* 99, 17095–17100.

Tuck-Muller, C.M., Narayan, A., Tsien, F., et al., 2000. DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. *Cytogenet. Cell Genet.* 89, 121–128.

van der Westhuyzen, J., 1985. Methionine metabolism and cancer. *Nutr. Cancer* 7, 179–183.

Velez-Garcia, E., Vogler, W.R., Bartolucci, A.A., Arkun, S.N., 1977. Twice weekly 5-azacytidine infusion in disseminated metastatic cancer: a phase II study. *Cancer Treat. Rep.* 61, 1675–1677.

Willar-Garea, A., Fraga, M.F., Espada, J., Esteller, M., 2003. Procaine is a DNA-demethylating agent with growth-inhibitory effects in human cancer cells. *Cancer Res.* 63, 4984–4989.

Weiss, A.J., Metter, G.E., Nealon, T.F., et al., 1977. Phase II study of 5-azacytidine in solid tumors. *Cancer Treat. Rep.* 61, 55–58.

Weiss, A., Keshet, I., Razin, A., Cedar, H., 1996. DNA demethylation in vitro: involvement of RNA [published erratum appears in *Cell* 95 (4) (1998) 573]. *Cell* 86, 709–718.

Wilks, A., Seldran, M., Jost, J.P., 1984. An estrogen-dependent demethylation at the 5'-end of the chicken vitellogenin gene is independent of DNA synthesis. *Nucleic Acids Res.* 12, 1163–1177.

Wu, J.C., Santi, D.V., 1985. On the mechanism and inhibition of DNA cytosine methyltransferases. *Prog. Clin. Biol. Res.* 198, 119–129.

Zhang, Y., Ng, H.H., Erdjument-Bromage, H., Tempst, P., Bird, A., Reinberg, D., 1999. Analysis of the NuRD subunits reveals a histone deacetylase core complex and a connection with DNA methylation. *Genes Dev.* 13, 1924–1935.

Zimmermann, F.K., Scheel, I., 1984. Genetic effects of 5-azacytidine in *Saccharomyces cerevisiae*. *Mutat. Res.* 139, 21–24.